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Genetic Relatedness and Molecular Characterization of

Multi-Drug Resistant *Acinetobacter baumannii*

By

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Genetic Relatedness and Molecular Characterization of Multi-Drug Resistant *Acinetobacter baumannii*

Ghiwa Makke

ABSTRACT

Acinetobacter baumannii is a Gram-negative organism associated with a broad range of severe nosocomial infections and linked to prolonged hospital stay of immunocompromised patients. We used whole-genome sequencing (WGS) to characterize 41 isolates of *A. baumannii* collected from a hospital in Lebanon. Resistance profiles were determined through antibiotic susceptibility testing (AST) and confirmed by the detection of resistance genes in the sequenced genomes. The plasmid content was analyzed both *in silico* and using the *Acinetobacter baumannii* – PCR based replicon typing (AB-PBRT) method. Multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and whole-genome SNP analysis were performed to investigate the possibility of an outbreak scenario and the relatedness between the isolates. Most of the isolates (95%; n=39) were resistant to cephalosporins, quinolones, aminoglycosides as well as to carbapenems, and were accordingly classified as being extensive drug resistant (XDR) and were referred to as carbapenem resistant *Acinetobacter baumannii* (CRAB). *in silico* analysis revealed the presence of 20 different resistance determinants each conferring resistance to one of the following: minoglycosides, β-lactams, sulfonamides, tetracyclines, or Macrolide-Lincosamide-Streptogramin (MLS). Gr2 (90%; n=37) and Gr6 (70.7%; n=29) plasmids were detected in 93% (n=38) of the isolates. ST-636 (48.7%; n=20), ST-2 (24.4%; n=10), ST-2-like (21.9%; n=9), ST- 396 (2.5%; n=1) and ST-388 (2.5%; n=1) were the only detected sequence types. All ST-636 isolates carried *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-72}}$, whereas ST-2 and ST-2-like isolates carried

bla$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-23}}$. To our knowledge, this is the first comprehensive whole-genome based characterization of CRAB associated with nosocomial infections in Lebanon, as well as in the Middle East. Our findings revealed that the studied MDR *A. baumannii* isolates were circulating in the hospital and were linked to two separate outbreaks during the same period infecting several immunocompromised patients and representing a leading cause of mortality.

Keywords: *Acinetobacter baumannii*, CRAB, WGS, PFGE, SNPs, outbreak.

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LIST OF ABBREVIATIONS

AB-PBRT: *Acinetobacter baumannii* PCR-based replicon typing

Acb: *A. calcoaceticus- A. baumannii*

AmpC: Class C β-lactamase

ARGs: Antimicrobial resistance genes

AST: Antibiotic susceptibility testing

CARD: Comprehensive antibiotic resistance database

CDC: Centers for Disease Control and Prevention

CRAB: Carbapenem resistant *A. baumannii*

DTA: Deep throat aspirate

ESBL: Extended-spectrum b-lactamases

HGT: Horizontal gene transfer

IC: International clones

ICU: Intensive care unit

IS: Insertion Sequence

LPS: Lipopolysaccharide

MDR: Multi-drug resistant

MLST: Multi-locus sequence typing

NJ: Neighbor-joining

OXA: Oxacillin hydrolyzing-β-lactamase

PBRT: PCR-based replicon typing

PCR: Polymerase chain reaction

PDR: Pan-drug resistant

PFGE: Pulsed-field gel electrophoresis

PLACNET: Plasmid Constellation Network

PT: Pulsotype

RAST: Rapid Annotation using Subsystem Technology

rep: replicase genes

ST: Sequence type

VF: Virulence factor

wg: Whole genome

WGS: Whole genome sequencing

XDR: Extensively drug resistant

MBL: metallo-β-lactamase

OMP: outer membrane proteins

ADC: *Acinetobacter*-Derived Cephalosporins

CHDL: carbapenem hydrolysing oxacillinases

MIC: minimal inhibitory concentrations

RND: resistance-nodulation-division

HCW: healthcare workers

Chapter One

Introduction

1.1 Overview of Acinetobacter baumannii

The *Acinetobacter* genus, belonging to the Family *Moraxellaceae* (Hu et al., 2013), consists of Gram-negative, strictly aerobic, non-fastidious, non-fermenting, non-motile, oxidase negative, catalase positive bacteria with 39 to 47% DNA GC content (Howard et al., 2012). The first organism was isolated in 1911, by the Dutch microbiologist Beijerinck, from soil using minimal media enriched with sodium acetate and was named *Micrococcus calcoaceticus* (Manchanda et al., 2010). However, the genus *Acinetobacter* was not proposed until 1954 by Brisou and Prevot, and was acknowledged in 1971 by the sub-committee on the Taxonomy of Moraxella and Allied Bacteria based on the publications of Baumann et al. (1968) (Howard et al., 2012). Members of the genus *Acinetobacter* are mostly environmental organisms not associated with human diseases (Gordon et al., 2010), but *A. baumannii* and some closely related species due to their pathogenic potentials have emerged as problematic nosocomial pathogens (Hu et al., 2013). The identification of species was via DNA-DNA hybridization method, however *A. baumannii* along with *A. calcoaceticus*, *A. pittii* (genomic species 3) and *A. nososcomialis* (genomic species 13TU) are phenotypically very similar to the extent that they are difficult to differentiate, and thus the term often used is "*A. calcoaceticus- A. baumannii*" complex (Acb complex) (Dijkshoorn et al., 2007).

1.2 Epidemiology and infections

Members of the *Acinetobacter* genus are ubiquitous in nature and are mostly isolated from soil and water. However, this is not true for the clinically important

species *A. baumannii* (Fournier et al., 2006). This organism is rarely found as part of the normal microbial flora of the skin (Howard et al., 2012), yet studies have shown that it can be recovered from the body lice of homeless people (La Scola & Raoult, 2004). *A. baumannii* is capable of causing both community and nosocomial infections (Fournier et al., 2006). Increasing reports of community acquired pneumonia have been associated with *A. baumannii*, and most of the cases are usually linked to underlying conditions such as alcoholism, old age, heavy smoking, diabetes mellitus and chronic obstructive pulmonary disease (Dijkshoorn et al., 2007). Other infections caused by community acquired *A. baumannii* might include skin and soft tissue infections, endocarditis and secondary meningitis (Antunes et al., 2014; Manachanda et al., 2010). When infecting skin and soft tissues, it can be distinguished by orange skin like appearance, which at later stages of the infection develops into sandpaper-like presentation. Vesicles as well as hemorrhagic bullae can be seen in areas of infected skin, if left untreated it might lead to septicemia and death (Howard et al., 2012).

A. baumannii also causes wound infections linked to natural disasters or conflict zones; the dry and sandy environment associated with such areas provide an ideal condition for the robustness of *A. baumannii* (Howard et al., 2012). *A. baumannii* also causes a wide range of nosocomial infections including: urinary tract, ventilator-associated pneumonia, wound and bloodstream in addition to community-acquired infections (Dijkshoorn et al., 2007; Antunes et al., 2014). These infections are a serious risk especially in the ICU, where patients are often chronically ill, immunocompromised, and hospitalized for extended periods (Howard et al., 2012). Other risk factors that might contribute to developing nosocomial infections linked to *A. baumannii* include: major surgery, prematurity in newborns, burn trauma, open

wounds, exposure to contaminated medical equipment such as catheters and mechanical ventilators (Dijkshoorn et al., 2007; Maragakis et al., 2008).

1.3 Antibiotic Resistance

A. baumannii has gradually developed to become a problematic pathogen due to the misuse of antibiotics and its ability to develop multidrug resistance (MDR) (Liu et al., 2014). In 2004, 70% of the isolates recovered from hospitals in the Middle East and Latin America were multidrug resistant (MDR) showing even resistance to carbapenems (Perez et al., 2007; Tognim et al., 2004). Resistance to carbapenem alone is enough to consider them as highly resistant (Poirel & Nordmann, 2006). In addition, there have been some reports of colistin resistance resulting in the emergence of strains resistant to all known antibiotics (Cai et al., 2012; Al-Sweih et al., 2011; Ko et al., 2007). *A. baumannii* is part of the ESKAPE pathogens, which are the leading cause of nosocomial infections (Howard et al., 2012). Eight clones of *A. baumannii* are spread worldwide and known as *A. baumannii* international clones (IC), of which IC1 and IC2 are MDR and commonly associated with hospital outbreaks (Salloum et al., 2018; Hua et al., 2017; Antunes et al., 2014).

1.3.1 Plasmids and Integrons

Depending on the antimicrobial resistance profile of *A. baumannii,* isolates showing resistance to at least three different classes of drugs (cephalosporins, fluoroquinolones and aminoglycosides) are classified as being multidrug-resistant (MDR). On the other hand, extensive drug resistant (XDR) isolates are those resistant to the former classes along with carbapenems, and pan drug resistant (PDR) those that are XDR and showing resistance to polymyxins (Manchanda et al., 2010).

Different resistance mechanisms were detected in *A. baumannnii* including intrinsic resistance mechanisms as well as foreign resistance determinants, which are acquired through mobile genetic elements such as plasmids and integrons (Dijkshoorn et al., 2007; Fournier et al., 2006). However, the MDR phenotype is mostly due to the latter (Adams et al., 2008).

A. baumannii plasmids range in size from 2 kb to more than 100 kb, and more studies should be done to fully understand their role. Plasmids are classified according to the nucleotide sequence similarities of the 27 identified replicase (*rep*) genes, and accordingly distributed into 19 homology groups (Lean et al., 2017). Resistance conferred by plasmids in *A. baumannii* is mostly correlated with the integrons they carry (Fournier et al., 2006). Integrons are conserved sequences capable of obtaining gene cassettes carrying antimicrobial resistance. The presence of integrons can be detected by the identification of integrin specific *int* gene, which encodes for the integrase (*att*) (Akrami et al., 2017). There are several types of integrons with the most common being class I and II. Class I integron carries resistance genes such as the: *dfr* (dihydroflavonol-4-reductase/trimethoprim), *sul1* gene (sulfonamide) and broad-spectrum β-lactamases encoding genes including carbapenemases (IMP, VIM, and even OXA-like genes) (Hou & Yang, 2015). Resistance determinants linked to class II are: *dfrA1*, *aadA1* (trimethoprim, streptomycin/ spectinomycin), *sat1* and *aadB* (streptothricin, kanamycin and tobramycin) (Akrami et al., 2017).

1.3.2 Carbapenem Resistance

Antimicrobial-inactivating enzymes such as β-lactamases are one of the important resistant mechanisms used by *A. baumannii* (Lee et al., 2017). Resistance to carbapenems is mainly related to the production of β-lactamases (Poirel & Nordmann, 2006). These enzymes are grouped into four classes (A, B, C and D). Class A β-lactamases hydrolyze penicillins and cephalosporins more efficiently than carbapenems except for KPC enzyme (Jeon et al., 2015). Of the enzymes belonging to this class, only SHV, TEM, CTX-M, GES, VEB, KPC, SCO, PER and CARB were identified in *A. baumannii*. Some variants of these enzymes are narrow-spectrum β-lactamases such as TEM-1, and CARB-4 while others are considered to be extended-spectrum β-lactamases (ESBLs) such as TEM-92, CTX-M-15, SHV-5 as well as PER-1. PER-1 was the first described ESBL in an *A. baumannii* isolate, which was recovered from Belgium (Lee et al., 2017; Jamal et al., 2018; Naas et al., 2006). Carbapenemases belonging to class A β-lactamases (KPC-2 and GES-14), however have been identified in *A. baumannii* isolates in Iran and France (Moubareck et al, 2009; Bogaerts et al., 2010 from Lee et al., 2017 Bonnin et al., 2011 and Azimi et al., 2015). Enzymes belonging to this class are mostly identified in *Enterobacteriaceae* as well as in *Pseudomonas aeruginosa*, but to a much lower extent in *A. baumannii* (AL-Marjani & Khadam, 2016). The second class is class B β-lactamases, which also plays an important role in carbapenem resistance (Poirel & Nordmann, 2006). Class B β-lactamases are metallo-β-lactamases (MBLs), which require heavy metal for the hydrolysis of a wide range of antibiotics covering carbapenems and excluding monobactams, and are inhibited via metal chelators (Jeon et al., 2015; Jamal et al., 2018; Lee et al., 2017), and often located on mobile genetic elements (Manchanda et al., 2010). MBLs were initially identified in *P.*

aeruginosa and were also reported later in *Enterobacteriaceae* and *A. baumannii* (Queenan and Bush, 2007). Out of the various enzymes belonging to this class, IMP, VIM, NDM and SIM have been identified in *A. baumannii.* IMP has to date nine variants detected in several isolates of *A. baumannii* recovered mainly from Asia, but also in South America and Europe. Conversely, both VIM and SIM were rarely detected in *A. baumannii* (Jamal et al., 2018; Roca et al., 2012; Potron et al., 2015; Poirel & Nordmann, 2006). Several variants of NDM, initially identified in *Enterobacteriaceae* recovered in India, were detected in *A. baumannii.* Class C β-lactamases, confer resistance to penicillins, cephalosporins and cephamycins. *A. baumannii* contains an intrinsic AmpC cephalosporin the overexpression of which and the resistance to extended spectrum cephalosporin is linked to the presence of an upstream IS known as *ISAba1* acting as a strong promoter (Howard et al., 2012; Lee et al., 2017). The variants of this enzyme are referred to as *Acinetobacter*-Derived Cephalosporins (ADC) since they were identified in *A. baumannii* (Jamal et al., 2018). Finally, the class D β-lactamases known as oxallinases (OXAs) is the most widespread carbapenemase, with more than 400 OXA-type enzymes being identified and categorized into subgroups termed carbapenem hydrolyzing oxacillinases (CHDLs). The most predominant subgroups are OXA-23, OXA-24, OXA-51 and OXA-58 (Lee et al., 2017). OXA-23 was first identified in *A. baumannii* isolated from UK in 1985, since then, it was detected worldwide being mainly associated with the wide dissemination of Carbapenem Resistant *Acinetobacter baumannii* (CRAB) (Perez et al., 2007; Mugnier et al., 2010; Al-Agamy et al., 2016; Chen et al., 2018). In Lebanon 76.5% of the *A. baumannii* isolates studied were carbapenem resistant of which 90% harbored OXA-23 (Al-Atrouni et al., 2016). OXA-24 on the other hand, was first identified in 1997 in an *A. baumannii* strain that was part of an

outbreak in Spain (Bou et al., 2006). Several variants of this enzyme have emerged including OXA-72, which were linked to nosocomial outbreaks in Spain, Ecuador and the United States (Chen et al., 2018; Evans & Amyes, 2014). OXA-51, is the largest group of intrinsic OXA-type β-lactamases identified. It was originally detected in 1996 in *A. baumannii* recovered from Argentina (Brown et al., 2005) and the gene became an important marker used for the identification of the organism at the species level (Howard et al., 2012). However, the presence of this gene does not lead to carbapenem resistance unless associated with an upstream IS (*ISAba1*) which would act as a strong promoter (Howard et al., 2012; Evans & Amyes, 2014). OXA-58 was first identified in France in 2003 (Poirel et al., 2005). Since then, it disseminated widely and has been linked to outbreaks in Europe, South America, Asia (Gusatti et al., 2012). In contrast to OXA-51, the presence of OXA-23, OXA-24, and OXA-58 is enough to confer resistance to carbapenems (Howard et al., 2012; Jamal et al., 2018).

1.3.3 Other mechanisms of carbapenem resistance

Efflux pumps are another important resistance determinants, which actively export many antimicrobial agents from the bacterial cell (Maragakis et al., 2008). AdeABC efflux system belonging to the resistance-nodulation-division (RND) superfamily is a chromosomally encoded and present in most *A. baumannii* strains (Dijkshoorn et al., 2007). It is linked to aminoglycoside resistance and to a decrease in susceptibility to tigecycline and non-fluoroquinolone drugs (Lee et al., 2017). However, resistance is only attained when point mutations in *adeS* gene or insertion of the *ISAba1* sequence upstream lead to overexpression of the pump (Dijkshoorn et al., 2007). AdeIJK and AdeFGH are other pumps from the same superfamily and are also associated with tigecycline resistance, whereas AbeM pump belongs to the

multidrug and toxic compound extrusion (MATE) family. This pump is also chromosomally encoded and its overexpression leads to imipenem and quinolones resistance (Dijkshoorn et al., 2007 and Lee et al., 2017). Major facilitator superfamily (MFS) on the other hand, includes TetA and TetB, which are linked to tetracycline and minocycline resistance (Gordon et al., 2010). This family also includes several novel efflux pumps such as AmvA associated with resistance to different classes of antibiotics, while the AbaF is associated with fosfomycin resistance (Lee et al., 2017). Other resistance mechanism encompasses porins, which are outer membrane proteins (OMPs) involved in cell permeability. The loss or modification of CarO protein, an OMP, is associated with the emergence of carbapenem resistance in *A. baumannii* due to the loss of imipenem binding site (Poirel & Nordmann, 2006; Mussi et al., 2007).

1.3.4 Treatment

Due to the rapid ability of *A. baumannii* to acquire antimicrobial resistance, only few effective drugs (carbapenems) are available for the treatment of MDR *A. baumannii* associated infections (Lee et al., 2017). However, the emergence of colistin-resistant *A. baumannii* resulting in strains resistant to all available antibiotics have limited treatment options (Antunes et al., 2014). Combination therapies such as colistin/imipenem, colistin/tigecycline and others have been studied as an alternative treatment option (Lee et al., 2017).

1.4 Virulence Factors

Despite of the clinical importance of this nosocomial pathogen, not much is known about its pathogenic potential or its virulence repertoire (Howard et al., 2012). Several factors are thought to contribute to the virulence potential of *A. baumannii* mediating environmental persistence along with host immunity evasion. Most of

these are encoded by genes that are part of the core genome such as the ones involved in adhesion and mobility, quorum sensing, iron metabolism and biofilm formation.

1.4.1 Capsule

The capsular polysaccharide encompasses the whole bacterium and protects it from any external threat, and thus is one of the factors contributing to the pathogenicity and survival of *A. baumannii.* The capsule allows the bacterium to counter the host complement system and help it to persist in the blood (Lee et al., 2017). It is an important factor where strains lacking the capsular polysaccharide were found to be avirulent (Harding et al., 2017). Other studies also have linked the capsule to the bacterium´s ability to resist prolonged periods of desiccation though direct evidence is still lacking (Harding et al., 2017). The different structural elements making up the capsule are encoded for by a chromosomal gene cluster known as the K locus; a highly conserved region followed by one that is highly variable and encoding for proteins involved in the biosynthesis of sugar precursors and transferases needed for the different capsular types (Eijkelkamp et al., 2014). *A. baumannii* has 25 distinct polysaccharide gene clusters, and the identification of the capsular gene can be determined by the sequencing of *wzy* gene (Hu et al., 2013). Capsular polysaccharides also have a role in the formation and maintenance of biofilms.

1.4.2 Outer Membrane Proteins

One of those factors is the outer membrane protein that is involved in cellular permeability. OmpA is one of the most abundant outer membrane protein, it binds to host epithelial cells and mitochondria inducing mitochondrial dysfunction followed by release of cytochrome c (a proapoptotic molecule), inducing apoptosis (Lee et al.,

2017). OmpA has a role in biofilm formation contributing to the persistence and survival of *A. baumannii* (McConnell et al., 2013), and in resistance to antimicrobial agents. The minimal inhibitory concentrations (MICs) for several antibiotics decreased following *ompA* disruption (Smani et al., 2014). The loss of CarO however, another outer membrane protein, was also shown to be associated with the emergence of carbapenem resistance in *A. baumannii* (Mussi et al., 2007).

1.4.3 LPS

The lipopolysaccharide (LPS) in *A. baumannii*, which is composed of an endotoxin lipid A moiety, carbohydrate core, but no O-antigen, is a major component of the outer membrane (Lee et al., 2017; Harding et al., 2017), playing a role in membrane integrity and stability (Harding et al., 2017). It also plays a major role in its survival and virulence, linked to septic shock once the bacterium enters the bloodstream, through activating the innate immune response (Fournier et al., 2006). Moreover, the lipid A moiety is the target of the last-line of treatment used with CRAB isolates. Unfortunately, many studies have shown that modifications or loss of the moiety could lead to resistance, decreasing the susceptibility of the organism to certain antimicrobial agents such as colistin by preventing its binding (Lee et al., 2017; Harding et al., 2017).

1.4.4 Pili

Medical-device-associated infections are associated with microbial biofilms. *A. baumannii* forms biofilms in the wound, on the wound dressing, as well as on abiotic surfaces of medical devices. Adhesins and capsular polysaccharides are associated with biofilm formation. Most strains have the Csu pili regulated by the BfmRS, two-component regulatory system (Tomaras et al., 2008). Csu pili are crucial for biofilm formation on abiotic surfaces such as catheters, ventilators and

other medical equipment, and have a role in bacterial motility (Harding et al., 2013). The twitching motility, which is used by *A. baumannii* for locomotion, relies on the type IV pili (Harding et al., 2013); genes encoding this type of pili are upregulated during bacterial growth in human serum indicating its possible involvement in bacteremia (Lee et al., 2017; Harding et al., 2017; Jacobs et al., 2012).

1.4.5 Siderophore

One of the problems faced by strictly aerobic bacteria is the ferric iron limitation. *A. baumannii* overcomes this limitation by a high affinity iron chelator known as a siderophore. The best-characterized and most common *A. baumannii* siderophore is the acinetobactin, which is considered a virulence factor since it increases the ability of the bacteria to persist within epithelial cells causing cell damage. In addition, the production of acinetobactin was shown to be more frequent in clinical MDR isolates than in avirulent isolates (Megeed et al., 2016).

1.4.6 Secretion Systems

Over the past few years, several protein secretion systems have been identified in *A. baumannii* including type I, type II, type IV, type V and type VI with the latter being the most important. Type VI (T6SS) is used by the bacterium to inject effector proteins in the host as well as to mediate the killing of competing bacteria. This system also may contribute to the spread of antibiotic resistance because it leads to DNA release and horizontal gene transfer (Borgeaud et al., 2015). On the other hand, some studies have shown that repressors of the T6SS might be carried on plasmids harboring resistance genes, the loss of which leads to a competent bacterium that is sensitive to antibiotics (Weber et al., 2015; Harding et al., 2017).

1.5 Transmission

Many factors are involved in the transmission and emergence of nosocomial outbreaks associated to *A. baumannii.* Although the ability of the bacterium to survive desiccation and persist on dry surfaces for a long period of time plays an important role in the transmission and emergence of outbreaks (D'Agata et al., 2000), contaminated medical equipment colonized with *A. baumannii* is considered the main reservoir for hospital outbreaks. In addition to that, several studies have showed that lack of compliance of healthcare workers (HCW) to the infection control recommendation contributed vastly in increasing cross-transmission between patients leading to outbreaks (Teerawattanapong et al., 2018; Wieland et al., 2018). Therefore, to control the spread of resistant strains and to limit the occurrence of outbreaks especially in ICU, periodic environment and patient surveillance as well as strict compliance to infection control precautions must be followed (Morgan et al., 2010; Markogiannakis et al., 2008).

1.6 Aims

The aims of this study were to:

- Investigate the antimicrobial resistance profile and identify the resistance gene reservoirs in the collected isolates
- Characterize the molecular determinants involved in virulence of these resistant pathogens
- Perform comparative analysis between different strains of *A. baumannii*
- Use genome sequence data to construct a genome-based phylogeny
- Examine and compare the genes present in the different clusters
    - Test for the relatedness of the isolates indicating a possible outbreak

Chapter Two

Materials and Methods

2.1 Specimen Collection

A total of 41 *A. baumannii* isolates were collected between April and December 2016 from hospitalized patients at a 544-bed governmental hospital in Lebanon and were designated as ACM-1 to ACM-37 based on the date of sample collection followed by ACM-38 to ACM-41 for the isolates with no trackable records. The 37 isolates were collected from 23 patients whose mean age was 53 ± 27 years old, ranging between newborn to 92 years old. Of these, 65% (n=15) were females and 35% (n=8) were males. 61% (n=14) of the patients in this study died due to various reasons including underlying diseases. *A. baumannii* isolation and identification was carried out inside the hospital using automated microbial identification system (Vitek, BD Pheonix).

Table 1. Demographic information including: type of specimen, date of isolation, patients' ID, patients' age and sex, and hospital ward and NCBI accession numbers. M, Male; F, Female; NB, newborn; ST, sequence type; DTA, deep tracheal aspiration; P fluid, peritoneal fluid; BW, bronchial wash; abdm, abdomen; umbl, umbilical; ICU, intensive care unit; ERICU, emergency room intensive care unit; CCU, coronary care unit; PED, pediatrics; NICU, neonatal intensive care unit; ONCO, oncology; Ped(OR), pediatrics operation room; Med1, medical floor 1; Med2, medical floor 2. '-' indicates that the information was not available.

Label	Specimen Type	Date of Isolation	Patient	Age	Sex	Ward	Accession #
ACM-1	DTA	4/9/2016	1	NB	F	NICU	MJAY000 00000
ACM-2	Eye culture	5/6/2016	2	NB	F	NICU	MJAZ000 00000
ACM-3	DTA	5/9/2016	3	81	F	ICU	MJBB000 00000
ACM-4	Blood	6/3/2016	4	66	F	ONCO	MJBA000 00000
ACM-5	DTA	6/4/2016	5	60	M	CCU	PYCX000 00000

ACM-6	BW	6/9/2016	6	59	F	ICU	MJBC000 00000
ACM-7	Surgical (abd)	6/11/2016	7	55	F	ICU	MJBD000 00000
ACM-8	DTA	6/15/2016	6	59	F	ICU	MJBE000 00000
ACM-9	DTA	6/17/2016	8	43	F	ERIC U	PYCV000 00000
ACM-10	Blood	7/29/2016	9	44	F	CCU	
ACM-11	DTA	7/29/2016	10	64	F	ICU	PYCW000 00000
ACM-12	BW	8/8/2016	11	47	M	ICU	PYCY000 00000
ACM-13	DTA	8/11/2016	12	69	M	MED 2	PYCZ000 00000
ACM-14	DTA	8/12/2016	13	71	F	ERIC U	PYDA000 00000
ACM-15	DTA	9/6/2016	14	52	F	ICU	PYDB000 00000
ACM-16	DTA	9/8/2016	15	70	F	ICU	PYDC000 00000
ACM-17	DTA	9/16/2016	16	59	F	ERCC U	PYDD000 00000
ACM-18	BW	9/19/2016	10	64	F	ICU	PYDE000 00000
ACM-19	Sputum	9/20/2016	16	59	F	ICU	PYDF000 00000
ACM-20	Surgical	9/22/2016	17	60	F	MED 2	PYDG000 00000
ACM-21	Surgical	9/22/2016	18	89	M	MED 1	PYDH000 00000
ACM-22	Blood	9/22/2016	19	3	M	PED	PYDI0000 0000
ACM-23	DTA	9/22/2016	20	65	M	ICU	PYDJ0000 0000
ACM-24	P. Fluid	9/23/2016	19	3	M	PED	PYDK000 00000
ACM-25	Blood	9/24/2016	19	3	M	PED	PXYP000 00000
ACM-26	P. Fluid	9/26/2016	19	3	M	PED	PYDL000 00000
ACM-27	DTA	9/27/2016	21	65	M	ICU	PYDM000 00000
ACM-28	Blood	9/27/2016	19	3	M	PED	PYDN000 00000
ACM-29	Peritoneal	9/28/2016	19	3	M	PED	PYDO000 00000
ACM-	Surgical	9/30/2016	19	3	M	PED	PYDP000

30						(OR)	00000
ACM-31	DTA	10/1/2016	18	89	M	MED 1	PYDQ000 00000
ACM-32	DTA	10/1/2016	20	65	M	MED 1	PYDR000 00000
ACM-33	Peritoneal	10/1/2016	19	3	M	PED	PYDS000 00000
ACM-34	Blood	10/4/2016	22	NB	F	NICU	QAGN000 00000
ACM-35	Surgical	10/5/2016	18	89	M	MED 1	QEHN000 00000
ACM-36	Surgical (umb)	10/7/2016	22	NB	F	NICU	QAGO000 00000
ACM-37	DTA	12/8/2016	23	92	M	ICU	QAGP000 00000
ACM-38	-	-	A	-	-	-	QAGQ000 00000
ACM-39	-	-	B	-	-	-	QAGR000 00000
ACM-40	-	-	C	-	-	-	QAGS000 00000
ACM-41	-	-	D	-	-	-	QAGT000 00000

2.2 Antimicrobial testing

Antimicrobial susceptibility testing was performed using disk diffusion method on Mueller-Hinton agar to determine the resistance patterns to 11 different antibiotics belonging to 5 categories: aminoglycosides (gentamicin and amikacin), quinolones (ciprofloxacin and levofloxacin), glycylcyclines (tigecycline), polymyxins (colistin), cephalosporins (ceftazidime and cefepime), carbapenems (imipenem and meropenem) and penicillin with β-lactamase inhibitor (piperacillin/tazobactam). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017)

2.3 Bacterial DNA extraction

Bacterial DNA extraction was performed using the Nucleospin® Tissue kit (Macherey-Nagel, Germany) from fresh bacterial colonies grown on TSA plates according to the manufacturer's instructions.

2.4 Plasmid Typing

PCR-based replicon typing method for *A. baumannii* developed by Bertini et al. (2010) was used to determine the plasmid content in the 41 isolates. In this method, the 27 known *A. baumannii* replicase (*rep*) genes are grouped into 19 homology groups according to their nucleotide sequence similarities.

Six multiplex PCR reactions were done using primers designed by Bertini et al. (2010).

Table 2. Homology groups clustered into 6 multiplex PCR, primer sequences and amplicon size.

Multiplex	Group	Primer	Sequence (5′-3′)	Ta (°C)	Amplicon size (bp)
1	GR1	gr1FW	CATAGAAATACAGCCTATAAAG	52	330
		gr1RV	TTCTTCTAGCTCTACCAAAAT		
	GR2	gr2FW	AGTAGAACAACGTTTAATTTTATTGGC	52	851
		gr2RV	CCACTTTTTTTAGGTATGGGTATAG		
	GR3	gr3FW	TAATTAATGCCAGTTATAACCTTG	52	505
		gr3RV	GTATCGAGTACACCTATTTTTTGT		
2	GR5	gr5FW	AGAATGGGGAACTTTAAAGA	52	220
		gr5RV	GACGCTGGGCATCTGTTAAC		
	GR18	gr18FW	TCGGGTTATCACAATAACAA	52	676
		gr18RV	TAGAACATTGGCAATCCATA		
	GR7	gr7FW	GAACAGTTTAGTTGTGAAAG	52	885
		gr7RV	TCTCTAAATTTTTCAGGCTC		
3	GR9	gr9FW	GCAAGTTATACATTAAGCCT	52	191
		gr9RV	AAAAATAAACGCTCTGATGC		
	GR4	gr4FW	GTCCATGCTGAGAGCTATGT	52	508
		gr4RV	TACGTCCCTTTTTATGTTGC		
	GR11	gr11FW	GGCTATTCAAAACAAAGTTAC	52	852
		gr11RV	GTTTCCTCTCTTACACTTTT		

4	GR12	gr12FW	TCATTGGTATTCGTTTTTCAAAACC	52	165
		gr12RV	ATTTCACGCTTACCTATTTGTC		
	GR10	gr10FW	TTTCACTAGCTACCAACTAA	52	371
		gr10RV	ACACGTTGGTTTGGAGTC		
	GR13	gr13FW	CAAGATCGTGAAATTACAGA	52	780
		gr13RV	CTGTTTATAATTTGGGTCGT		
5	GR8	gr8FW	AATTAATCGTAAAGGATAATGC	52	233
		gr8RV	GACATAGCGATCAAATAAGC		
	GR14	gr14FW	TTAAATGGGTGCGGTAATTT	52	622
		gr14RV	GCTTACCTTTCAAAACTTTG		
	GR15	gr15FW	GGAAATAAAAATGATGAGTCC	52	876
		gr15RV	ATAAGTTGTTTTTGTTGTATTCG		
6	GR16	gr16FW	CTCGAGTTCAGGCTATTTTT	52	233
		gr16RV	GCCATTTCGAAGATCTAAAC		
	GR17	gr17FW	AATAACACTTATAATCCTTGTA	52	380
		gr17RV	GCAAATGTGACCTCTAATATA		
	GR6	gr6FW	AGCAAGTACGTGGGACTAAT	52	662
		gr6RV	AAGCAATGAAACAGGCTAAT		
	GR19	gr19FW	ACGAGATACAAACATGCTCA	52	815
		gr19RV	AGCTAGACATTTCAGGCATT		

2.5 Pulsed-field gel electrophoresis (PFGE)

Plugs with *A. baumannii* genomic DNA were prepared according to protocol developed by Seifert et al. (2005). Briefly, bacterial DNA was digested using the restriction enzyme *ApaI* (Thermo Fisher Scientific, MA, USA) for 2 h at 37ºC. DNA fragments were then separated on 1% Seakem Gold gel using Bio-Rad Laboratories CHEF DR-III system (Bio-Rad Laboratories, Inc, CA, USA) for 16 h with initial and final switch time of 7s and 20s respectively. *Salmonella enterica subsp. enterica serovar Braenderup* (ATCC® BAA664TM) was used as reference for band size and *XbaI* restriction 0enzyme (Thermo Fisher Scientific, MA, USA) was used for the digestion. The gel was then stained with ethidium bromide and viewed under UV. Fingerprints were then analyzed using BioNumerics software version 7.6.1 (Applied Maths, St-Martens-Latem, Belgium) and a dendogram was generated to show the relatedness of the isolates. Different pulsotypes (PT) were assigned when a difference in three or more bands was detected (Tenover et al., 1995).

2.6 Whole-genome sequencing and assembly

Library preparation was done using the Illumina Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). 1 µg of each isolate's genomic DNA (gDNA) was used as an input. According to the manufacturer's protocol, each gDNA was subjected to end-repair, A-tailing, ligation of adaptors including sample-specific barcodes. Quantification was done using Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The sequencing of the library was then done on an Illumina MiSeq using pair end 500-cycle protocol to read a length of 250 bp. Genome assembly was performed

de novo assembly was done using Spades Genome Assembler Version 3.9.0 (Bankevich et al. 2012), and quality control checks of the raw sequences using FastQC version 1.0.0 (Andrews et al., 2010).

2.7 Genome Annotation

The assembled genomes were annotated using RAST server (http://rast.nmpdr.org) (Aziz et al., 2012). For the identification of acquired antibiotic resistance genes, Resfinder (CGE- www.genomicepidemiology.org.) was used with a 98% identity threshold (ID) (Zankari et al., 2012). Virulence factors were determined through RAST search against Virulence Factor Data Base (VFDB). The Multilocus sequence type (MLST) was determined using the MLST 1.8 server available on goseqit database (CGE- www.genomicepidemiology.org) (Larsen et al., 2012). IS-Finder (https://www-is.biotoul.fr/) was used to identify the insertion sequences (Siguier et al., 2006). For plasmid identification using raw reads, the Plasmid Constellation Network (PLACNETw) was used (Lanza et al., 2014).

2.8 wg-SNP-based phylogenetic analysis

SNP-based analysis was performed on the BioNumerics v7.6.1 software (Applied Maths, Sint-Martens-Latem, Belgium) using the strict SNP filtering options that are recommended to use for SNP analysis within outbreak situations. The strict SNP filtering removes positions with at least one unreliable (N) base, ambiguous (non-ACGT) bases or gaps. It removes non-discriminatory positions and each retained SNP has a minimum of 5x coverage, at least covered once in both forward and reverse direction and with a minimum distance of 12 bp between retained SNPs. A maximum Likelihood tree was constructed from the obtained SNPs.

Chapter Three

Results

3.1 Isolate typing

MLST was performed for sequence based molecular typing of the isolates along with *in silico* MLST analysis based on seven house-keeping genes (*cpn60, fusA, gltA, pyrG , recA, rplB,* and *rpoB*). The results obtained revealed the presence of five different STs among the 41 *A. baumannii* isolates. The most common ST detected in 49% (n=20) of the isolates was ST-636 (allelic profile 2-1-2-2-2-1-1). ST-2 was detected in 25% (n=10) of the isolates and had an allelic profile of 2-2-2-2-2-2-2. ST-2 differed from the ST-2-like, detected in 22% (n=9) of the isolates, by an Adenine to Thymine substitution (nucleotide 281) in the *fusA* gene. Only one isolate was typed as ST-388 (1-62-3-2-40-4-4) (2%) and another as ST-396 (60-21-46-10-20-18-20) (2%). (Figures 2-4-5)

3.2 Susceptibility profiling

Antibiotic susceptibility typing was done on the 41 *A. baumannii* isolates. 92.8% (n=38) of the isolates were non-susceptible to both gentamicin and amikacin, in addition to one isolate (ACM-13) showing intermediate resistance phenotype for amikacin while being susceptible to gentamicin. Majority however showed quinolone resistance with 95% (n=39) being resistant to ciprofloxacin, and 92.8% (n=38) to levofloxacin and one isolate (ACM-3) showing intermediate resistance. High resistance was also detected (95%; n=39) to: ceftazidime (cephalosporin), imipenem and meropenem (carbapenems), piperacillin/tazobactam (penicillin with β-lactamase inhibitor), and 82.9% (n=34) of the isolates to cefepime (cephalosporin), and 4.87% (n=2) showing intermediate resistance to cefepime. The lowest detected

resistance was against tigecycline (glycylcycline 26.8%; n=11), while all were susceptible to colistin. Based on the above 95% of the isolates in this study were classified as being extensive drug resistant (XDR) (resistant to cephalosporins, quinolones, aminoglycosides and carbapenems) (Figure 1).

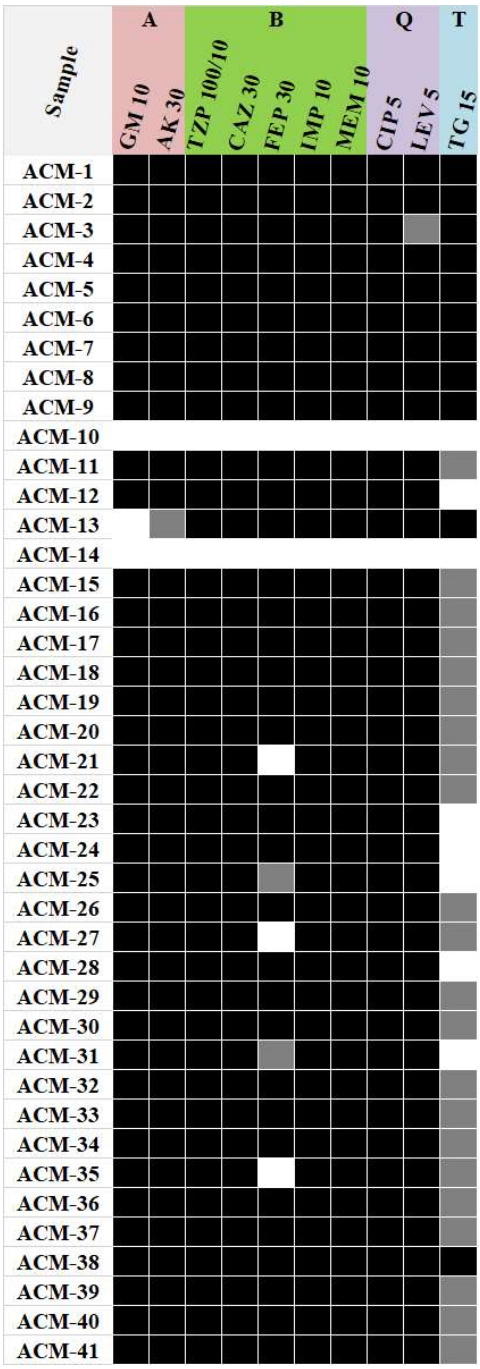


Figure 1. Antimicrobial susceptibility profiles of the isolates undertaken in this study. Classes of antibiotics are marked as follows: A, aminoglycosides; B, β-lactams; Q, Quinolones; T, tetracyclines. GM, gentamicin; AK, amikacin; TZP, Piperacillin/Tazobactam; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MEM, meropenem; CIP, ciprofloxacin; LEV, levofloxacin; TG, tigecycline. Black indicates 'resistant', grey indicates 'intermediate susceptibility' and blank indicates 'sensitive'.marked as follows: A, aminoglycosides; B, β-lactams; Q, Quinolones; T, tetracyclines. GM, gentamicin; AK, amikacin; TZP, Piperacillin/Tazobactam; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MEM, meropenem; CIP, ciprofloxacin; LEV, levofloxacin; TG, tigecycline. Black indicates 'resistant', grey indicates 'intermediate susceptibility' and blank indicates 'sensitive'.

3.3 Resistance genes

The antibiotic susceptibility typing results were additionally confirmed through *in silico* detection of resistant determinants via ResFinder. Four different aminoglycoside inactivating enzymes in addition to their variants were found in the sequenced genomes. Aminoglycoside acetyltransferase (ACC) variant *aac(3')-Ia* and *ant(3'')-Ia* belonging to aminoglycoside nucleotidyltransferase (ANT) family were detected in 56% (n=23) and 53.6% (n=22) of the isolates, respectively. Five variants of the aminoglycoside phosphotransferase (APH) enzyme were also identified. The most common was *aph(3')-VIa* and was found in 73% (n=30) of the isolates. *strA* and *strB* variants of *aph* enzyme were detected in 46.3% (n=19) of the isolates, *aph(3')-Ia* in 34% (n=14), *aph(6')-Id* in 2.4% (n=1), and *armA* detected in 46.3% (n=19) of the isolates.

We used different tools to investigate the presence of intrinsic and/or acquired β-lactam linked resistant determinants. Core chromosome genes, $bla_{\text{ADC-25}}$ (*Acinetobacter*-Derived Cephalosporinase) and $bla_{\text{OXA-51}}$ through its two variants $bla_{\text{OXA-66}}$ and $bla_{\text{OXA-71}}$, were found in all the isolates. Other Carbapenem-Hydrolyzing Class D β-Lactamases (CHDLs) such as: $bla_{\text{OXA-72}}$ ($bla_{\text{OXA-24-like}}$), $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-359}}$ were seen in 60.9% (n=25), 43.9% (n=18) and 2.4% (n=1) of the isolates, respectively. Class A β-lactamase ($bla_{\text{TEM-1D}}$) in addition to macrolide resistant determinants *mph(E)* and *msr(E)* were respectively detected in 31.7% (n=13) and 44% (n=18) of the isolates (Figure 2).

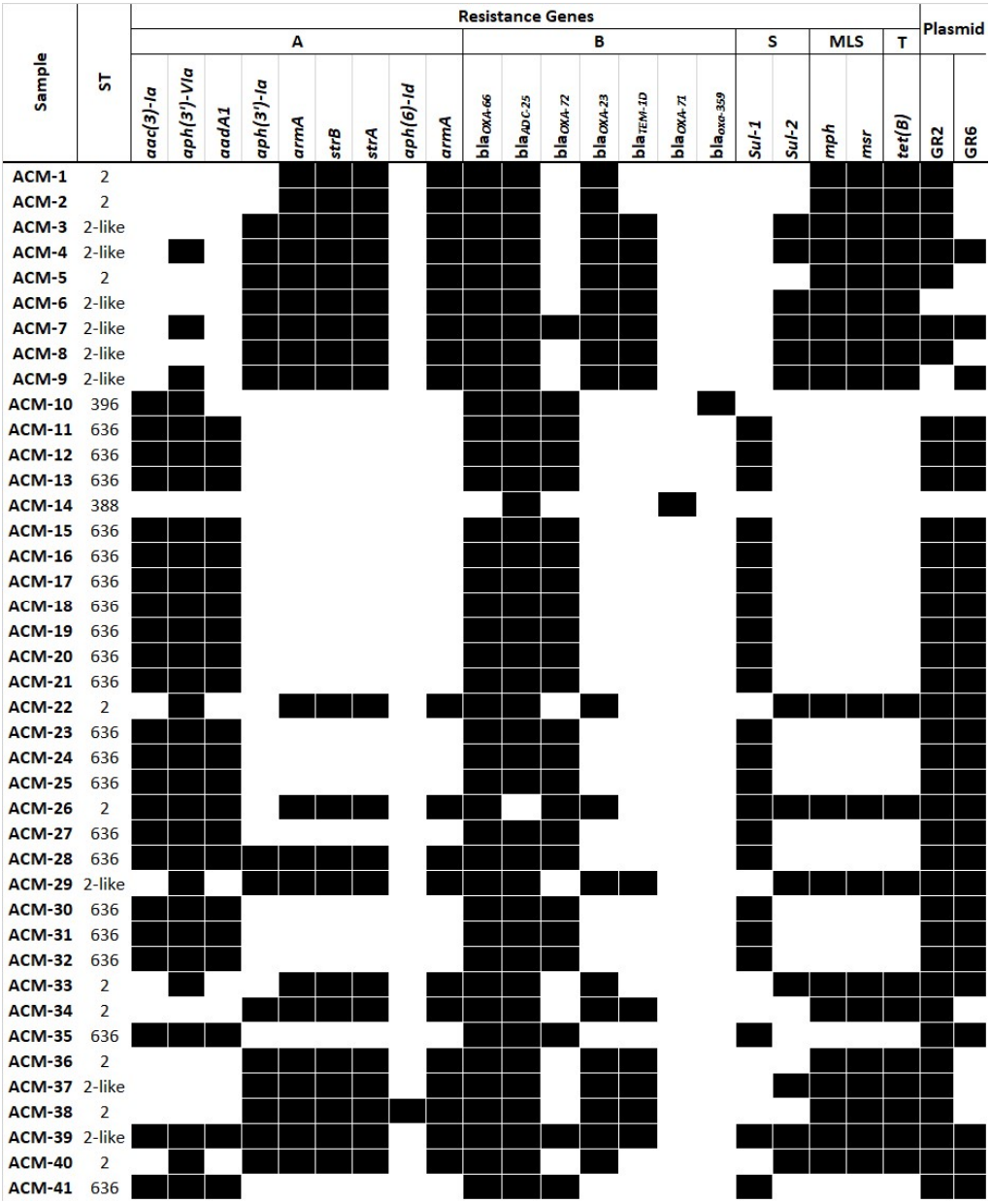


Figure 2. Sequence types, antibiotic resistance genes, and homology groups detected by PCR and in silico. ST, Sequence type. Classes of antibiotic resistance genes are designated as follows: A, aminoglycoside resistance genes; B, β-lactam resistance genes; S, sulfonamide resistance genes; MLS, macrolide-lincosamide-streptogramin resistance genes; T, tetracycline resistance genes.

3.4 Homology group typing

Using *Acinetobacter baumannii* PCR-based replicon typing (AB-PBRT), two homology groups GR-2 and GR-6 were detected in 90.2% (n=37) and 70.7% (n=29) of the isolates, respectively, while 68% (n=28), showed both GR-2 and GR-6. Nine of the isolates (21.9%) contained only GR-2, only one (ACM-9) contained GR-6, and three (ACM-6, ACM-10 and ACM-14) had none (Figure 2).

3.5 Virulence Factors

Several factors contribute to the virulence potential of *A. baumannii*. Outer membrane protein encoding genes linked to cellular permeability were detected in all of the isolates including: *ompA*, *ompH*, *ompYfgL* and *ompImp*. Adhesion is another important virulence determinant with type IV pili, type 1 fimbriae and sigma fimbriae seen in all sequenced genomes in addition to type 1 secretion system (T1SS). Siderophores such as anthrachelin and achromobactin were among the detected virulence determinants found in 56% (n=23) and 97.5% (n=40) of the isolates. Anthrachelin, and in contrast to ST-2, ST-2-like, ST-388 and ST-396, was only detected in 10% (n=2) of isolates typed as ST-636 (ACM-30 and ACM-35) (Figure 3).

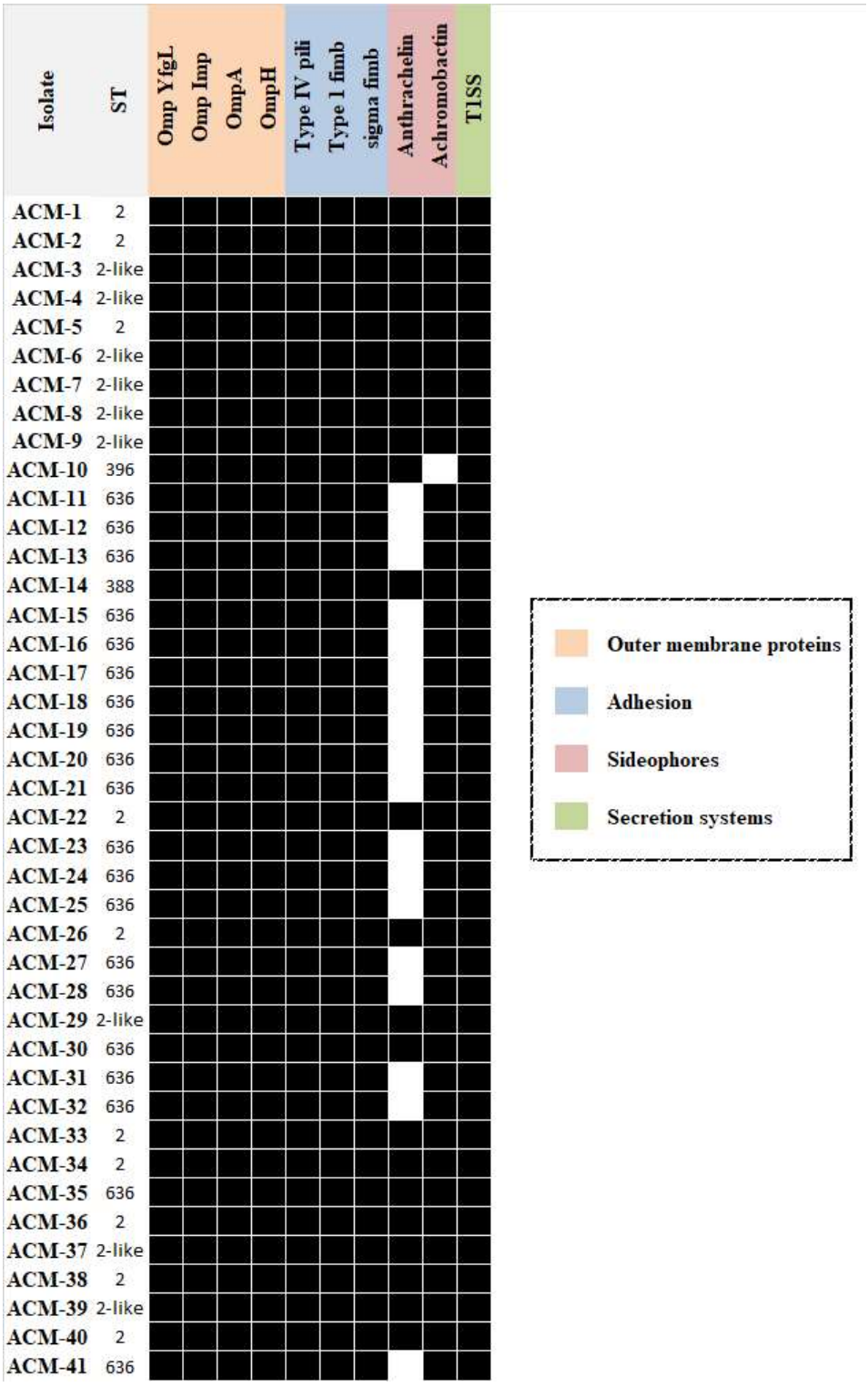
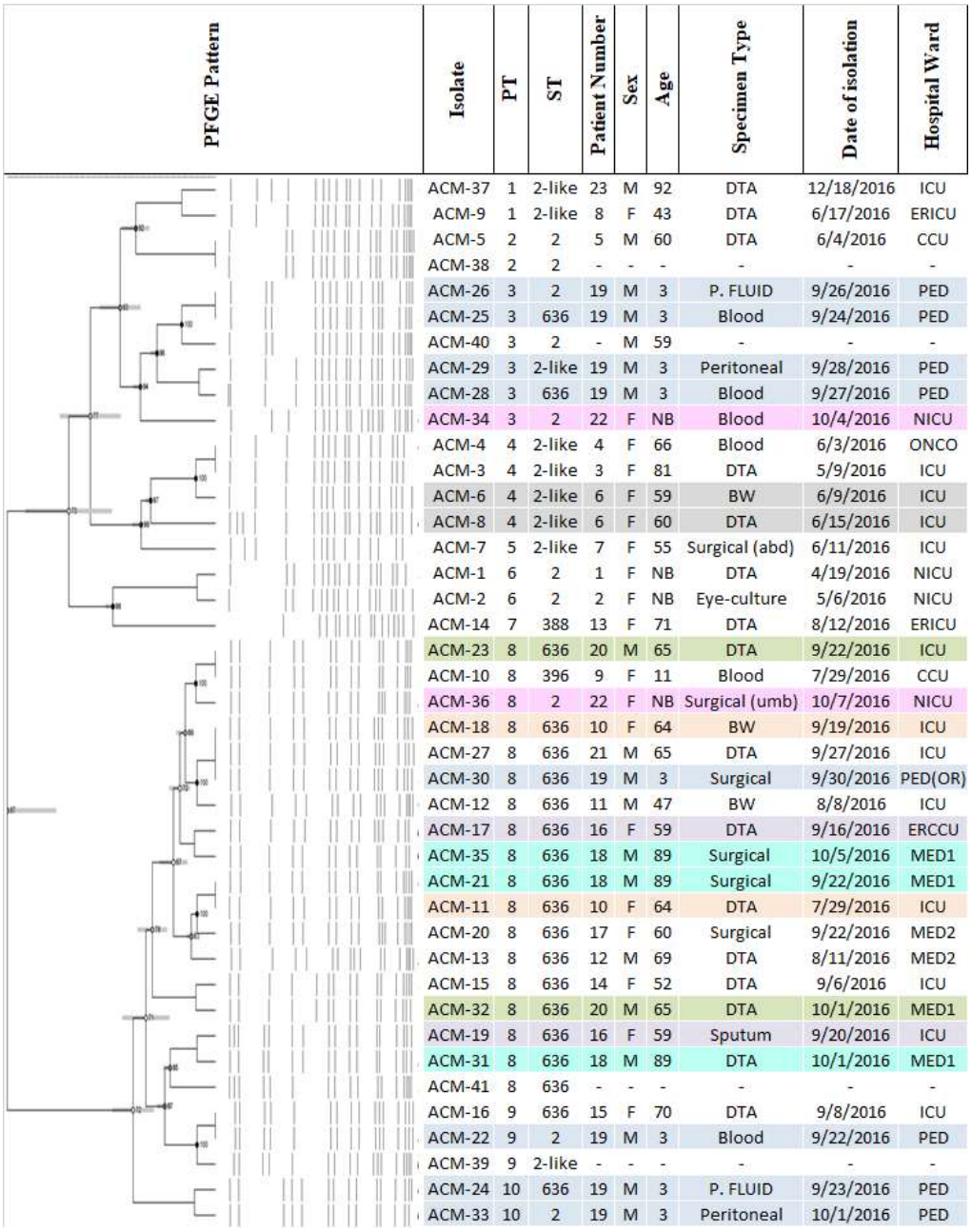


Figure 3. Virulence determinants detected in the 41 *A. baumannii* isolates. Black indicates presence of the virulence factor, and blank indicates absence of virulence factor. ST, sequence type. Outer membrane proteins include: ompA, ompH, ompYfgL and ompImp. Adhesions include: type IV pili, type 1 fimbria and sigma fimbria. Siderophores include: anthrachelin and achromobactin. Secretion systems include: T1SS, type 1 secretion system.

3.6 PFGE

Ten different pulsotypes (PTs) were identified showing a difference in more than three bands (Tenover et al., 1995). The isolates were clustered into two major clades that didn't show any correlation to the sequence type or specimen source. The first clade included in total 18 isolates divided into 7 pulsotypes. These isolates were mainly of ST-2 and ST-2-like (Figure 4). In contrast, the second clade, which had the remaining 23 isolates distributed within three pulsotypes. The majority of these isolates were of ST-636. Some of the isolates having the exact same PT and same ST, after close investigation, were found to be isolated in the same time-frame, same patient or same hospital ward. ACM-26 (ST-2) and ACM-25 (ST-636), were recovered from a three-year old child, 2 days apart, but from different sites (peritoneal fluid and blood). On the other hand, ACM-3 (ST-2-like) and ACM-4 (ST-2-like) were recovered from different infection sites (DTA versus blood), different patients, and different hospital wards but yet showed the exact same banding pattern. ACM-23 (ST-636) and ACM-10 (ST-396; later identified as *A. calcoaceticus*) were collected from different patients, hospital wards, and time-frame, yet interestingly also had same banding patterns (Figure 4). Furthermore, ACM-16 (ST-636), isolated from DTA of a 70-year-old female patient admitted to the ICU, had a 100% match to ACM-22 (ST-2) which was recovered from blood of a 3-year-old male patient admitted to the pediatric ward. Also, ACM-30 (ST-636), ACM-27 (ST-636) and ACM-18 (ST-636) isolated within a ten days period of time from different patients at different hospital ward had a 100% similarity in PFGE.



PFGE Pattern	Isolate	PT	ST	Patient Number	Sex	Age	Specimen Type	Date of isolation	Hospital Ward
	ACM-37	1	2-like	23	M	92	DTA	12/18/2016	ICU
	ACM-9	1	2-like	8	F	43	DTA	6/17/2016	ERICU
	ACM-5	2	2	5	M	60	DTA	6/4/2016	CCU
	ACM-38	2	2	-	-	-	-	-	-
	ACM-26	3	2	19	M	3	P. FLUID	9/26/2016	PED
	ACM-25	3	636	19	M	3	Blood	9/24/2016	PED
	ACM-40	3	2	-	M	59	-	-	-
	ACM-29	3	2-like	19	M	3	Peritoneal	9/28/2016	PED
	ACM-28	3	636	19	M	3	Blood	9/27/2016	PED
	ACM-34	3	2	22	F	NB	Blood	10/4/2016	NICU
	ACM-4	4	2-like	4	F	66	Blood	6/3/2016	ONCO
	ACM-3	4	2-like	3	F	81	DTA	5/9/2016	ICU
	ACM-6	4	2-like	6	F	59	BW	6/9/2016	ICU
	ACM-8	4	2-like	6	F	60	DTA	6/15/2016	ICU
	ACM-7	5	2-like	7	F	55	Surgical (abd)	6/11/2016	ICU
	ACM-1	6	2	1	F	NB	DTA	4/19/2016	NICU
	ACM-2	6	2	2	F	NB	Eye-culture	5/6/2016	NICU
	ACM-14	7	388	13	F	71	DTA	8/12/2016	ERICU
	ACM-23	8	636	20	M	65	DTA	9/22/2016	ICU
	ACM-10	8	396	9	F	11	Blood	7/29/2016	CCU
	ACM-36	8	2	22	F	NB	Surgical (umb)	10/7/2016	NICU
	ACM-18	8	636	10	F	64	BW	9/19/2016	ICU
	ACM-27	8	636	21	M	65	DTA	9/27/2016	ICU
	ACM-30	8	636	19	M	3	Surgical	9/30/2016	PED(OR)
	ACM-12	8	636	11	M	47	BW	8/8/2016	ICU
	ACM-17	8	636	16	F	59	DTA	9/16/2016	ERCCU
	ACM-35	8	636	18	M	89	Surgical	10/5/2016	MED1
	ACM-21	8	636	18	M	89	Surgical	9/22/2016	MED1
	ACM-11	8	636	10	F	64	DTA	7/29/2016	ICU
	ACM-20	8	636	17	F	60	Surgical	9/22/2016	MED2
	ACM-13	8	636	12	M	69	DTA	8/11/2016	MED2
	ACM-15	8	636	14	F	52	DTA	9/6/2016	ICU
	ACM-32	8	636	20	M	65	DTA	10/1/2016	MED1
	ACM-19	8	636	16	F	59	Sputum	9/20/2016	ICU
	ACM-31	8	636	18	M	89	DTA	10/1/2016	MED1
	ACM-41	8	636	-	-	-	-	-	-
	ACM-16	9	636	15	F	70	DTA	9/8/2016	ICU
	ACM-22	9	2	19	M	3	Blood	9/22/2016	PED
	ACM-39	9	2-like	-	-	-	-	-	-
	ACM-24	10	636	19	M	3	P. FLUID	9/23/2016	PED
	ACM-33	10	2	19	M	3	Peritoneal	10/1/2016	PED

Figure 4. PFGE profiles, ST, patient number, specimen type, date of isolation, ward of the 41 *A. baumannii* isolates. PT, pulsotype; M, Male; F, Female; NB, newborn; ST, sequence type; DTA, deep tracheal aspiration; P fluid, peritoneal fluid; BW, bronchial wash; abdm, abdomen; umbl, umbilical; ICU, intensive care unit; ERICU, emergency room intensive care unit; CCU, coronary care unit; PED, pediatrics; NICU, neonatal intensive care unit; ONCO, oncology; Ped(OR), pediatrics operation room; Med1, medical floor 1; Med2, medical floor 2. '-' indicates that the information was not available. The dendogram was generated using BioNumerics software version 7.6.1. showing the phylogenic relatedness between the isolates. The restriction enzyme used to generate the banding pattern was *ApaI*.

3.7 wgSNP-based phylogenetic analysis

In total, 1,162,003 intergenic mutations were detected between all the isolates and the four used reference genomes (ST-2 and ST-636). The wgSNP-based phylogenetic tree showed two major clades (Figure 5), which was in accordance with the STs of the isolates. The first clade contained all the ST-636 that clustered with the two used ST-636 reference genomes (A105 and A074 of accession numbers SAMN03577730 and SAMN03576358 respectively). The second clade clustered together the ST-2, ST-2-like isolates (except for ACM-26 which had a separate line of descent), and the two used ST-2 reference genomes (ABBL008 and ZQ2 of accession numbers LLCO00000000 and PHKA00000000 respectively). ACM-14 (ST-388) and ACM-10 (ST-396) had on the other hand, separate lines of descent, with ACM-10 being later identified as *A. calcoaceticus* (Figure 5).

SNP analysis of the ST-636 isolates revealed the presence of 55 SNPs, with the highest number (n=15) being detected in ACM-31. ACM-17, ACM-19 and ACM-32 had identical SNPs. ACM-17 was isolated from the DTA of 59 year-old female patient admitted to ERCCU while ACM-19 was isolated 4 days later from the sputum of same patient who was transferred to the ICU, whereas ACM-32 was collected also from the DTA but from a different patient (65 year-old male) admitted to a different hospital ward (Med 1) 10 days later. Similarly, ACM-30, ACM-25 and ACM-28 collected from the same 3-year-old child during 1 week from peritoneal catheter insertion and blood did not show any polymorphisms (Figure 5).

Polymorphisms in ST-2 and ST-2-like isolates were also tracked, and 169 SNPs were detected in total. ACM-7 had the largest number of SNPs (n=143). Both ACM-40 and ACM-26 had 28 retained SNPs, although ACM-26 had a separate line of descent. Only two SNPs were retained between ACM-36 and ACM-34, which

recovered from the umbilical cord and blood, respectively of a newborn child, three days apart. The SNPs were present in a hypothetical protein and an AraC family transcriptional regulator.

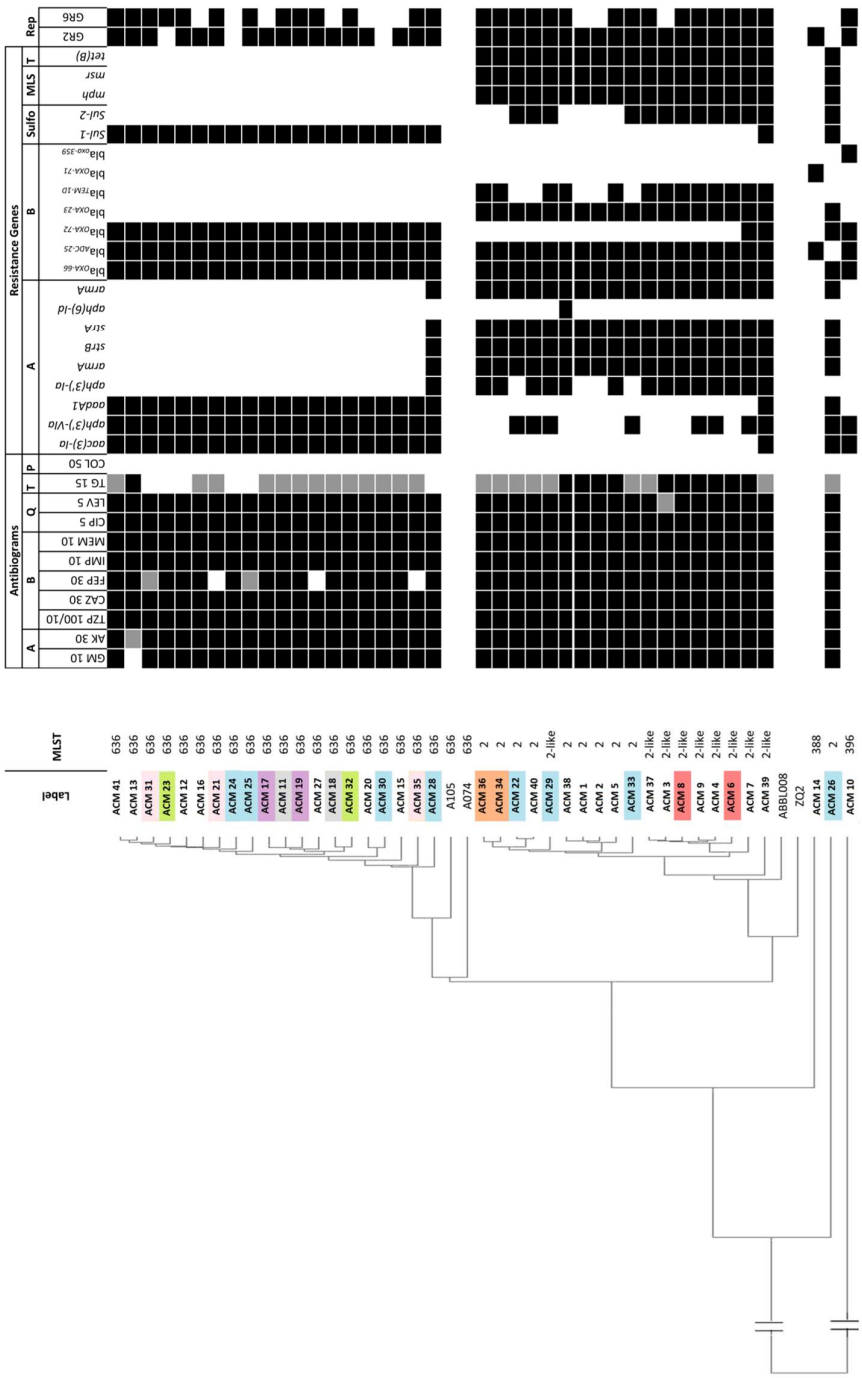


Figure 5. wgSNP-based phylogenetic tree, ST, antibiograms, antibiotic resistance genes, and replicon groups of the 41 isolates. ST, Sequence type. Classes of antibiotics are designated as follows: A, Aminoglycosides; B, β-lactam; Q, Quinolone; T, Tertracycline; P, Polymixin. Classes of antibiotic resistance genes are designated as follows: A, aminoglycoside resistance genes; B, β-lactam resistance genes; S, sulfonamide resistance genes; MLS, macrolide-lincosamide-streptogramin resistance genes; T, tetracycline resistance genes. wgSNP-based phylogenetic tree was generated using BioNumerics software version 7.6.1.

Chapter Four

Discussion

A. baumannii isolated from hospital environments over the past decade has been recognized to be multi-drug resistant (Agodi et al., 2014). This is mostly due to its ability to persist and colonize, in addition to its increasing resistance to most available antibiotics including carbapenems making it a threat to patients (Handal et al., 2017). Carbapenem-resistant *A. baumannii* (CRAB) has been recognized as a source of outbreaks worldwide by The American Centers for Disease Control and Prevention (CDC) (Handal et al., 2017), being associated with increasing morbidity and mortality rates in intensive care units (ICU) (Rafei et al., 2014).

This study aimed at the molecular characterization of 41 multi-drug resistant *A. baumannii* isolates collected between April 2016 and December 2016 from 23 patients admitted to different wards at a hospital in Lebanon. The rate of carbapenem-resistance detected in this study, was higher than what was previously reported from other countries such as Saudi Arabia (69%) and Italy (45.7 %) (Al Sultan et al., 2015 and Principe et al., 2014) or even from Lebanon (87%) (Moghnieh et al., 2019). Resistance to carbapenems was confirmed phenotypically and through the detection of carbapenemase encoding genes $bla_{\text{OXA-72}}$ ($bla_{\text{OXA-24-like}}$) and $bla_{\text{OXA-23}}$, which are the main worldwide carbapenem-resistance determinants in *A. baumannii* (Al Atrouni et al., 2016).

CRAB has been globally linked to outbreaks (Handal et al., 2017), and several typing techniques were used to discriminate and confirm possible hospital outbreaks (Fitzpatrick et al, 2016). In this study, we used PFGE, WGS and MLST

analysis to follow on a possible CRAB linked nosocomial outbreak. Our results revealed two main clones associated with the high incidence of *A. baumannii* linked infections, namely ST-636 and ST-2. ST-636 was the most common identified ST in this study (49%; n=20) and has previously been reported to be the cause of CRAB linked nosocomial infection in Sweden (Karah et al, 2016), and Tunisia (Mathlouthi et al, 2018). ST-636 was also reported previously in Lebanon but not as taking part of an outbreak (Al Atrouni et al., 2016). WGS analysis of ST-636 isolates showed that they had similar set of virulence factors and resistance determinants in all the isolates except for ACM-28, which had four additional aminoglycosides resistance genes (*aph(3')-Ia, armA, strB* and *strA*).

ST-2 and ST-2-like were detected in 25% (n=10) and 22% (n=9) of the isolates respectively, and showed 0.16% difference in the *fusA* gene sequence mainly linked to the nucleotide 281, where Adenine was substituted with Thymine. ST-2 linked to the international clone IC-II (Fitzpatrick et al, 2016), was previously linked to hospital outbreaks (Antunes et al, 2014) in Italy (Agodi et al, 2014), Greece (Pournaras et al, 2017), Thailand (Molter et al., 2015), and in Lebanon (Al Atrouni et al, 2016). WGS analysis of ST-2 and ST-2-like isolates in this study revealed that both had similar patterns of resistance against aminoglycosides, β-lactams, and sulfonamides, whereas resistance determinants detected in ST-2 were different from that detected in ST-636 isolates. In addition ST-2 and ST-2-like isolates had resistant determinants against macrolides and tetracycline.

ACM-10 and ACM-14, which were recovered within the same period of time and typed as ST-396 and ST-388 respectively, were not part of the circulating outbreak strains in the hospital and were also previously detected in India and Malaysia (Rynga et al., 2015; Biglari et al, 2017). Both carried fewer resistant

determinants. Knowing however, the potential of *A. baumannii* to readily acquire resistance determinants (Al Atrouni et al., 2016), we consider the two as potential future outbreak candidates.

ACM-10, isolated from blood of a 44 years-old female patient admitted to the CCU due to cardiac arrest resulting in brain anoxia injury, was first misidentified to be *A. baumannii*, but WGS, revealed that it was a misidentified *A. calcoaceticus*. Although *A. calcoaceticus* was mainly considered as being an environmental species, several reports revealed that it could cause pneumonia and bacteremia (Mostachio et al., 2012 and Jia et al., 2015). In Lebanon, Rafei et al. (2015) isolated non-*baumannii Acinetobacter* species including *A. calcoaceticus* from various food sources, while Al Atrouni et al. (2016) isolated carbepenem resistant *A. calcoaceticus* from vegetables.

Using the conventional band-based typing approach, PFGE, 10 different pulsotypes (PT) were detected, with different PTs encompassing isolates showing differences in more than three bands (Tenover et al., 1995). The isolates were clustered into two major clades that didn't show any correlations with the ST, specimen source, or hospital ward. Unlike other bacteria, genomically identical *A. baumannii* isolates might end up having different PFGE based banding patterns, which sometimes could be misleading especially within an outbreak scenario (Kanamori et al., 2016; Salipante et al., 2015). Some of the isolates in this study recovered from the same patient at different time intervals showed different banding patterns. ACM-22, ACM-24, ACM-25, ACM-26, ACM-28 ACM-29, ACM-30, and ACM-33 were isolated from blood and peritoneal fluid of a three-years old male patient within a period of 10 days. The isolates were of three different sequence types namely: ST-2, ST-2-like and ST-636. ACM-25 (ST-636) and ACM-26 (ST-2) had

the same PFGE banding patterns. This and other similar findings revealed the possibility of cross-transmission between patients and hospital wards.

The success of *A. baumannii* within the nosocomial setting is mostly due to its genome plasticity, which enabled the organism to survive under increased stress and environmental diversity by gaining genetic elements or undergoing genomic modifications leading to the accumulation of SNPs (Bogaty et al., 2018). Several studies confirmed that typing based on wgSNPs provided a higher discriminatory power and helped in better separating and clustering the isolates (Alshahni et al., 2015; Kanamori et al., 2016). In this study, the wgSNP-based phylogenetic tree showed two major clades, which were in accordance with the STs of the isolates. The first clade included all ST-636 isolates while the second included ST-2 and ST-2-like isolates. SNP analysis revealed the presence of 55 SNPs within the ST-636 isolates, while it was 169 for ST-2 and ST-2-like isolates. This relatively low number of detected SNPs could be attributed to outbreak-linked isolates where the changes indicate shorter time-span (Kanamori et al., 2016). ACM-36 and ACM-34 where different in only two nucleotides, both being recovered three days apart from umbilical cord and blood of a newborn girl. The SNPs were detected in a hypothetical protein and in an AraC family transcriptional regulator. This family of transcriptional regulators, are spread among different bacteria and regulate genes having diverse functions, ranging from carbon metabolism, stress responses, or virulence (Frota et al., 2004).

To our knowledge, several previous reports from Lebanon have shown CRAB was involved in nosocomial outbreaks, however this is the first comprehensive whole-genome based characterization of CRAB linked to nosocomial infections in Lebanon, as well as in the Middle East. Our findings revealed that the

studied MDR *A. baumannii* isolates were circulating the hospital and that were associated with two separate outbreaks during the same period of time infecting several immunocompromised patients and representing a leading cause of mortality where 61% of the patients (n=14) died as a result of the associated infection. Our data reconfirms that WGS is the best available tool for rapid detection of circulating outbreaks in hospital settings. Knowing that CRAB outbreaks is nowadays considered a threat to patients and thus a public health concern, several measures should be taken to limit the spread of this pathogen throughout the hospitals. We are currently investigating the genetic environment of the recovered plasmids, and closing the gaps in the draft genome to have closed plasmids with a full sequence and accurate annotations. In parallel we are doing more detailed phylogenetic analysis using different tools to better understand and elucidate whether the two outbreaks can be clearly split into isolates carrying plasmid mediated $bla_{\text{OXA-23}}$ and another linked to isolates having the *ISAba1* upstream of $bla_{\text{OXA-66}}$.

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